PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :		(11) International Publication Number: WO 92/08493
A61K 45/05, 37/10, C07K 13/00	A1	(43) International Publication Date: 29 May 1992 (29.05.92)

(21) International Application Number: PCT/US91/08212

(22) International Filing Date: 5 November 1991 (05.11.91)

(30) Priority data:
620,221 8 November 1990 (08.11.90) US

(71) Applicant: BRIGHAM AND WOMEN'S HOSPITAL [US/US]; 75 Francis Street, Boston, MA 02115 (US).

(72) Inventors: GOLDBERG, Mark, A. ; 96-2 Anderer Lane, Boston, MA 02132 (US). FAQUIN, William, C. ; 18 Anson Street, Boston, MA 02130 (US).

(74) Agents: FOX, Samuel, L. et al.; Sterne, Kessler, Goldstein & Fox, 1225 Connecticut Avenue, N.W., Suite 300, Washington, DC 20036 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.

(54) Title: USE OF INTERLEUKIN-6 TO STIMULATE ERYTHROPOIETIN PRODUCTION

(57) Abstract

The invention is directed to the administration of Interleukin 6 for the purpose of enhancing endogenous erythropoietin levels *in vitro* and *in vivo*. The invention further encompasses methods of treatment of disorders characterized by, or including, erythropoietin deficiency. Such disorders include the anemias of chronic inflammation, renal failure, AIDS, and malignancy.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland			SN	Senegal
CI	Côte d'Ivoire	KR	Republic of Korea	SU+	Soviet Union
CM	Cameroon	LI	Liechtenstein	TD	Chad
CS	Czechoslovakia	LK	Sri Lanka	TG	Togo
DE*	Germany	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		

+ Any designation f "SU" has effect in the Russian Federation. It is not yet known whether any such designati n has effect in ther States of the f rmer Soviet Union.

TITLE OF THE INVENTION

USE OF INTERLEUKIN-6 TO STIMULATE ERYTHROPOIETIN PRODUCTION

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to the use of interleukin-6 to stimulate the production of endogenous erythropoietin in cells *in vitro* and *in vivo*. The invention further includes methods of treatment wherein interleukin-6 is administered to humans and animals having disorders which are manifested by erythropoietin deficiency. The invention also includes methods of producing erythropoietin *in vitro*.

2. Description of the Background Art

The Biology of Erythropoietin

Early investigations by Reissmann, using a parabiotic rat model (Reissmann, K.R., Blood 5:372-80 (1950)), provided strong evidence for the humoral regulation of erythropoiesis. Later studies by Erslev (Erslev, A., Blood 8:349-57 (1953)), provided further evidence that a hormone, an erythropoietin, was involved in the regulation of red blood cell production. Subsequent studies were undertaken attempting to purify this hormone and to understand the mechanism by which its production is physiologically regulated.

To achieve the above aims, it became important to determine the sites of erythropoietin production. In 1956 and 1957, Jacobsen and his colleagues determined that the kidney is the primary site of erythropoietin production (Fried, W., et al., Proc Soc Exp Biol Med 92:203-7 (1956); Jacobson, L.O., et al., Nature 179:633-4 (1957)). Many subsequent in vivo studies and studies in isolated kidney preparations have confirmed that the kidneys are the principal site of erythropoietin production in adult mammals (Kuratowska, Z., et al., Blood 18:527-34 (1961); Erslev, A.J., Am J Med 58:25-30 (1975); Fisher, J.W., et al., Blood 29:114-25 (1967)). This finding is clearly illustrated by the following observations: (a) serum erythropoietin levels are lower than would be expected for the degree of anemia seen in patients with end-stage renal disease (Caro, J., et al., J Lab Clin Med 93:449-58 (1979)) and (b) the anemia or renal failure can be completely alleviated by the administration of recombinant human erythropoietin (Eschbach, J.W., et al., N Engl J Med 316:73-8 (1987); Winearls, C.G., et al., Lancet 2:1175-8 (1986)).

Although the investigations of Jacobsen clearly demonstrated that the kidney plays a central role in the production of erythropoietin, Nathan et al. (Nathan, D.G., et al., J Clin Invest 43:2158-65 (1964)) demonstrated regulation of erythropoiesis in an anephric man. In addition, Fried et al. (Fried et al., J Lab Clin Med 73:244-8 (1969)), demonstrated the presence of erythropoietin in the serum of nephrectomized rats. The production of erythropoietin was regulated by the degree of hypoxia to which the animals were exposed. Hence it appeared that the kidney was not the sole organ capable of producing

erythropoietin. Subsequently, the role of the adult liver as an erythropoietin-producing organ, particularly in times of stress, has been confirmed by multiple investigators (Naughton, B.A., et al., Science 196:301-2 (1977); Caro, J., et al., Am J Physiol 244:E431-4 (1983); Beru, S., et al., Mol Cell Biol 7:2571-5 (1986); Bondurant, M., et al., Mol Cell Biol 6:2731-3 (1986)). Other studies have shown that the liver is the primary site of erythropoietin production in the fetus (Zanjani, E.D., et al., J Lab Clin Med 83:281-7 (1974); Zanjani, E.D., et al., J Lab Clin Med 89:640-4 (1977); Zanjani, E.D., et al., J Lab Clin Med 67:1183-8 (1981); Flake, A.W., et al., Blood 70:542-5 (1987)). The switch in the primary site of erythropoietin production from the liver to the kidney occurs gradually beginning the last third of the gestational period, and is completed approximately 40 days after birth (Zanjani, E.D., et al., J Lab Clin Med 67:1183-8 (1981)); Flake, A.W., et al., Blood 70:542-5 (1987)).

Although the physiologically important erythropoietin-sensitizing organs are believed to be the kidney and the liver, there have been reports of erythropoietin production by a subpopulation of murine bone marrow macrophages (Rich, I.N., et al., Blood 60:1007-18 (1982); Vogt, C., et al., Exp Hematol 17:391-7 (1989)). Finally, various tumors have been reported to secrete erythropoietin or an erythropoiesis-stimulating activity. This production of erythropoietin has been most commonly reported to occur in renal tumors, primarily renal cell carcinomas (Kazal, L.A., et al., Ann Clin Lab Sci 5:98-109 (1975); Hagiwara, M., et al., Blood 63:828-35 (1984);

Sherwood, J.B., et al., Endocrinology 99:504-10 (1976); Sherwood, J.B., et al., Proc Natl Acad Sci USA 83:165-9 (1986)) and hepatomas (Davidson, C.S., Semin Hematol 13:115-9 (1976)); however, it has also been associated with uterine fibromyomas (Naets, J.P., et al., Scand J Haematol 19:75-8 (1977)), cerebellar hemangioblastomas (Waldmann, T.A., et al., Ann NY Acad Sci 149:509-15 (1968)), aldosterone secreting adrenyl adenomas (Mann, D.L., et al., Ann Int Med 66:335-40 (1967)), and pheochromocytomas (Bradley, J.E., et al., J Urol 86:1-6 (1961)).

Regulation of Erythropoietin Production in a Cell Line

Both the HepG2 and Hep3B cell lines produce large amounts of biologically active and immunologically identifiable erythropoietin in response to hypoxia or cobalt chloride. RNA blot analysis has demonstrated that the production of erythropoietin in these cells is regulated at the level of mRNA. These two cell lines were generated from human hepatic carcinomas (Aden, D.P., et al., Nature 282:615-6 (1979)); they have been shown histologically and biochemically to possess characteristics of well-differentiated liver parenchymal cells (Aden, D.P., et al., Nature 282:615-6 (1979); Knowles, B.B., et al., Science 209:497-9 (1980)). Both have many of the biosynthetic capabilities of normal hepatocytes, and they have been shown to secrete 17 of the major plasma proteins into cell culture medium, including albumin and alpha-fetoprotein. Further evidence suggesting that this cell line accurately reflects the normal physiological situation in vivo is provided by RNA blot hybridization analysis. The erythropoietin mRNA size

is indistinguishable from that previously reported for RNA from normal human fetal liver (Jacobs, K., et al., Nature 313:806-10 (1985)). In addition, the finding that regulation occurs at the mRNA level is in agreement with previous in vivo data (Beru, S., et al., Mol Cell Biol 7:2571-5 (1986); Bondurant, M., et al., Mol Cell Biol 6:2731-3 (1986)).

RNA blot analyses from hypoxic and nonhypoxic kidney tissue have shown that erythropoietin synthesis in the mouse kidney is regulated at the level of RNA (Beru, S., et al., Mol Cell Biol 7:2571-5 (1986); Bondurant, M., et al., Mol Cell Biol 6:2731-3 (1986)). Erythropoietin synthesis in the human hepatoma cell line, Hep3B, is also regulated at the RNA level, with a greater than 100-fold increase in erythropoetic mRNA levels after the cells have been made hypoxic or exposed to cobalt chloride for 24 hours (Goldberg, M.A., et al., Science 242:1412-5 (1988)). Furthermore, protein synthesis was necessary prior to hypoxia or cobalt-induced increase in erythropoietin mRNA in the Hep3B cells. Similar findings have been reported in hypoxic mouse kidneys (Schuster, S.J., et al., Blood 70:316-8 (1987)). Recently, Goldberg et al. (Goldberg, M.A., et al., Blood 74:716A (1989)) have found only a five- to ten-fold increase in the rate of erythropoietin gene transcription in Hep3B cells exposed to hypoxia or cobalt and have presented data suggesting a significant post-transcriptional component to the regulation of erythropoietin mRNA levels as well.

In Vivo Regulation of Erythropoietin Production

The maintenance of an adequate supply of oxygen to the body tissues is vital to survival. Since to a large degree the oxygen-carrying capacity of blood is governed by the concentration of erythrocytes in the blood, the appropriate regulation of erythropoiesis is also crucial. The early studies of Reissmann (Reissmann, K.R., Blood 5:372-80 (1950)) and Erslev (Erslev, A., Blood 8:349-57 (1953)) clearly demonstrated the hypoxia-induced stimulation of erythropoietin secretion. There are no preformed stores of erythropoietin in either the liver or the kidney, and hence hypoxia results in de novo production of erythropoietin (Jelkmann, W., Pflügers Arch 393:88-91 (1982); Schooley, J.C., et al., Blood 40:662-70 (1972)). Fried et al. (Fried, W., et al., Proc Soc Exp Biol Med 92:203-7 (1956)) noted that erythropoietin production was regulated by the relationship between oxygen supply and demand. An important point from these studies is that erythropoietin production appears to be regulated by the tissue oxygen tension at the site of the erythropoietin oxygen sensor in the kidney and liver. (Reissmann, K.R., Blood 5:372-80 (1950); Erslev, A., Blood 8:349-57 (1953); Fried, W., et al., Proc Soc Exp Biol Med 92:203-7 (1956); Jelkmann, W., Pflügers Arch 393:88-91 (1982); Schooley, J.C., et al., Blood 40:662-70 (1972)).

Erythropoietin Mechanism of Action: Target Cells

Unlike several of the other hematopoietic growth factors, erythropoietin is a lineage-specific

hematopoietin, only influencing the growth of cells of the erythroid lineage. When erythropoietin is secreted from the erythropoietin-producing cells in response to hypoxia, it travels through the blood to its target organ, the hematopoietic tissues; in man, before birth, the principal hematopoietic tissue is within the liver, while after birth it is in the bone marrow. There, erythropoietin binds specifically to its receptor on the erythroid progenitor cells called burst forming unit-erythroid (BFU-E) and colony-forming unit-erythroid (CFU-E) and stimulates these cells to proliferate and differentiate (Spivak, J.L., Int J Cell Cloning 4:139-66 (1986)). BFU-E are the earliest erythroid progenitors and constitute 0.01%, approximately, of the nucleated bone marrow cells. CFU-E, which are derived from BFU-E, and account for about 0.1% of marrow cells, are much more responsive to erythropoietin (Spivak, J.L., Int J Cell Cloning 4:139-66 (1986); Sawada, K., et al., J Clin Invest 80:357-66 (1987)).

Recent studies (Koury, M.J., et al., J Cell Physiol 121:526-32 (1984)) have described the population of cells obtained from the spleens of mice during the acute disease caused by infection with the anemia-inducing strain of the Friend murine leukemia virus. In an elegant series of investigations, it was demonstrated that these relatively immature erythroid cells require erythropoietin in order to maintain their viability and to differentiate into mature erythrocytes (Koury, M.J., et al., J Cell Physiol 121:526-32 (1984); Koury, M.J., et al., Proc Natl Acad Sci USA 79:635-9 (1982); Koury, M.J., et al., J Cell Physiol 126:259-65 (1986); Bondurant, M.C., et al., Mol Cell Biol 5:675-80 (1985); Sawyer, S.T., et al.,

J Biol Chem 261:9187-95 (1986); Koury, M.J., et al., J Cell Physiol 137:65-74 (1988); Koury, M.J., et al., Blood Cells 13:217-26 (1987); Koury, M.J., et al., J Cell Physiol 133:438-48 (1987)). During differentiation in response to erythropoietin, these cells increase globin gene transcription and synthesis of hemoglobin, transfer and receptor synthesis, and the synthesis of integral erythrocyte membrane proteins. They also undergo other normal differentiation-associated events such as enucleation and extensive membrane rearrangement.

Erythropoietin Levels in Health and Disease

The low levels of erythropoietin that are always present appear to be sufficient to allow for a basal rate of erythropoiesis. Relatively small losses of blood do not appear to stimulate increased erythropoietin production (Kickler, T.S., et al., J Am Med Assoc 260:65-7 (1988)). It is only after a major blood loss that there is an increased production of erythropoietin and rate of erythropoiesis.

It has been well-established that the majority of patients with renal insufficiency and anemia have serum erythropoietin levels well below what would be expected for the degree of anemia (Caro, J., et al., J Lab Clin Med 93:449-58 (1979); Radtke, H.W., et al., Blood 54:877-84 (1979); Chandra, M., et al., J Pediatr 113:1015-21 (1988)), although they can still respond to hypoxia with an increase in serum erythropoietin levels (Radtke, H.W., et al., Blood 54:877-84 (1979); Chandra, M., et al., J Pediatr 113:1015-21 (1988)). However, this markedly blunted erythropoietin response substantially contributes to the pathogenesis of the

anemia (Eschbach, J.W., et al., Am J Kid Dis 11:203-9 (1988)).

With the current availability of accurate immunoassays, investigators have begun to survey anemias of diverse etiologies to determine if the erythropoietin response is appropriate. There is a wide range of normal erythropoietin responses to anemia and different types of anemia may elicit different yet adequate degrees of erythropoietin response. Erslev et al. (Erslev, A.J., et al., J Lab Clin Med 109:429-33 (1987)) and Birgegard et al. (Birgegard, G., et al., Br J Haematol 65:479-83 (1987)) found that the erythropoietin response in anemic patients with rheumatoid arthritis and other inflammatory arthridities appear to be similar to the response in other types of anemias without active inflammation. On the other hand, the study by Baer et al. (Br J Haematol 66:559-64 (1987)) suggests that the erythropoietin response in patients with rheumatoid arthritis and anemia is blunted compared to a control population of anemias of other idiologies (including iron deficiency, malignancy, sickle cell anemia, pure red cell aplasia, aplastic anemia, pernicious anemia, sideroblastic anemia secondary to alcoholism, and other chronic, inflammatory disorders. Hochberg, MC, et al. (Arthritis and Rheumatism 31:1318-21 (1988)) also reported that patients with rheumatoid arthritis have an impaired serum immunoreactive erythropoietin response to anemia compared to a control group with iron deficiency anemia. Similarly, there are reports of blunted erythropoietin responses for a given degree of anemia in patients with cancer (Miller, C.B., et al., Proc Am Soc Clin Oncol 8:182 (1989)), AIDS (Spivak, J.L., et al. J Am Med Assoc 261:3104-7

(1989)), thalassemia (Manor, D., et al., Scand J Haematol 37:221-8 (1986)), sickle cell anemia (Sherwood, J.B., et al., Blood 67:46-9 (1986)), and the anemia of prematurity (Brown, M.S., et al., J Pediatr 105:793-8 (1984)), as well as in patients who have received cis-platinum-containing chemotherapy (Smith, D.H., et al., Proc Am Assoc Canc Res 29:52 (1988)) and in those who have received intensive radiotherapy and/or chemotherapy while undergoing bone marrow transplantation (Schapira, L., et al., Blood 74:910a (1989)). Furthermore, although most patients with myelodysplastic syndromes and anemia have very high serum erythropoietin levels, there are occasional patients with unexplained extremely low levels for the degree of anemia (Jacobs A. et al., Brit J. Haematol 73:36 (1989)).

In the case of sickle cell anemia, erythropoietin deserves special mention. Sherwood et al. (Sherwood, J.B., et al., Blood 67:46-49 (1986)) suggest that sickle cell anemia patients have low erythropoietin levels for their degree of anemia, although clearly these patients have a markedly increased rate of erythropoiesis and generally maintain reticulocyte counts in the range of 10-20%.

The Biology of Interleukin-6

The function of most cytokines that are known to be involved in the regulation of the immune response and hematopoiesis is not limited to a specific lineage of cells. These cytokines display a variety of biological functions on various tissues and cells. Interleukin-6 is a typical example of a multi-functional cytokine. The fact that this cytokine regulates immune responses, hematopoiesis, and acute phase reactions, indicates that it has a major role in host defense mechanisms.

Originally, human interleukin-6 was identified as B cell growth factor-2 or BSF2. It was found in the culture supernatants of mitogen- or antigen-stimulated peripheral mononuclear cells and shown to induce immunoglobulin production in B cell lines (Muraguchi, A., et al., J Immunol 127:412 (1981)). This factor was distinguishable from other factors, such as interleukin-2, 4, and 5 (other factors that affect B cells). BSF2 was purified to homogeneity from the culture supernatant of a human T cell leukemia virus type-1 (HTLV-1) transformed T cell line and its partial N-terminal amino acid sequence was determined (Hirano, T., et al., Proc Natl Acad Sci USA 82:5490 (1985)).

Around the time of the studies of BSF2, the nucleotide sequences of the molecules designated as interferon beta-2 (IFNβ_2) (Zilberstein, A., et al., EMBO 5:2529 (1986)) and 26 Kd protein were reported (Haegeman, G., et al., Eur J Biochem 159:625 (1986)). The sequencing studies revealed that BSF2, IFNβ_2, and 26 Kd protein were identical proteins. Subsequently, recombinant interleukin-6 was shown to lack any

interferon activity and to have no antigenic or functional relation to IFNβ (Hirano, T., et al., Immunol Lett 17:41 (1988)).

In addition to the function of interleukin-6 in the immune response, the cytokine functions in the induction of acute phase proteins in hepatocytes where it induces the production of major acute phase proteins (Gauldie, J., et al., Proc Natl Acad Sci USA 84:7251 (1987); Andus, T., et al., FEBS Lett 221:18 (1987)). Table 1 summarizes the molecules identified to be identical to interleukin-6.

Table 1

Molecules Identical to Interleukin-6

1. B cell stimulatory factor 2
2. Interferon beta 2
3. 26 Kd protein
4. Myeloma/plasmacytoma growth factor
5. Hepatocyte stimulating factor
6. Macrophage granulocyte inducing factor 2
7. Cytotoxic T cell differentiation factor

Immune system: Originally, interleukin-6 was found to be produced by T cells and shown to play a role in the induction of the late maturation of B cells into antibody-producing cells (Muraguchi, A., et al., J Immunol 127:412 (1981); Hirano, T., et al., Proc Natl Acad Sci USA 82:5490 (1985)). Interleukin-6 was also effective in in vivo antibody production in mice (Takatsuki, F., et al., J Immunol 141:3072 (1988)). A study with anti-interleukin-6 antibody demonstrated that interleukin-6 is one of the essential factors for antibody production in B cells (Muraguchi, A., et al., J Exp Med 167:332 (1988)).

The effect of interleukin-6 is not restricted to B cells, however. It can also act on T cells.

Activated but not resting B cells express interleukin-6 receptors while resting T cells express interleukin-6 receptors (Taga T., et al., J Exp Med 166:967 (1987)). This result indicates that interleukin-6 acts only on the final maturation stage of activated B cells and is effective on resting T cells. In the presence of interleukin-2, interleukin-6 was also shown to induce differentiation of cytotoxic T cells (CTL) from murine and human thymocytes and spleen. (Takai, Y., et al., J Immunol 140:508 (1988); Okada, M., et al., J Immunol 141:1543 (1988)).

Acute phase Reactions: The acute phase response is a systemic reaction to inflammation or tissue injury that is characterized by leukocytosis, fever, increased vascular permeability, alterations in plasma metal and steroid concentrations, and increased levels of acute phase proteins. Several acute phase proteins are induced by interleukin-6, such as fibrinogen, alpha-1-antichymotrypsin, alpha-1-acid glycoprotein, and haptoglobin in human hepatoma cell line, HepG2. Serum amyloid A, C-reactive protein, and alpha-1-antitrypsin in human primary hepatocytes are also induced by interleukin-6 (Castell, J.V., et al., FEBS Lett 232:347 (1988)). In vivo administration of interleukin-6 in rats also induced characteristic acute phase reactions. The results confirmed the in vivo role of interleukin-6 in acute phase reaction (Geiger, T., et al., Eur J Immunol 18:717 (1988)).

Interleukin-6 Signals: Interleukin-6 provides multiple signals on various tissues and cells. These signals can be divided into three categories: (a) induction of differentiation or specific gene

expression such as immunoglobulin induction in B cells, induction of acute phase proteins in hepatocytes, induction of cytotoxic T cell differentiation, induction of neural cell (PC12) differentiation, and activation of hematopoetic stem cells from G_0 to G_1; (b) stimulation of cell growth, such as the induction of myeloma/plasmocytoma growth or T cell growth, and induction of mysangial cell growth; (c) inhibition of cell growth such as inhibition of growth of myeloid leukemia cells or breast cancer cells.

Hematopoiesis: The effect of interleukin-6 on hematopoiesis was first described by Ikebuchi et al. (Ikebuchi, K., et al., Proc Natl Acad Sci USA 84:9035 (1987)). It was found that interleukin-3 and interleukin-6 act synergistically in the formation of multi-lineage blast cell colonies in murine spleen cell cultures. The appearance of these cell colonies induced by IL-3 was significantly hastened by the addition of interleukin-6, suggesting that interleukin-6 activates hematopoetic stem cells at the G_0 stage to enter into the G_1 phase.

Human and murine myeloid leukemia cell lines such as human histiocytic U937 cells and mouse myeloid M1 cells can be induced to differentiate into macrophages and granulocytes in vitro by several synthetic and natural products. Recently, interleukin-6 was also shown to induce differentiation of M1 cells into macrophages (Miyaura, C., et al., FEBS Lett 234:17 (1988)).

Recent studies by a number of different laboratories have demonstrated that interleukin-6 affects the growth and/or differentiation of a

diversity of hematopoietic cell types. In vitro studies assessing the effect of interleukin-6 on bone marrow progenitor cells from adult bone marrow and fetal cord blood demonstrated that interleukin-6 induced cycling and clonogenic maturation of CFU-Mix, CFU-GM, and BFU-E (Gardner et al., Blood 75:2150-2155 (1989)). Other in vitro studies in which growth of hematopoietic precursors was measured, showed that combinations of interleukin-6 and either M-CSF, GM-CSF, G-CSF, IL-3, or IL-4 enhanced the total number and/or colony size of erythroid, myeloid, megakaryocytic, and multilineage colonies (Bot, et al., Blood 73:435-437 (1989); Rennick, D. et al., Blood 73:1828-1835 (1989)).

Interleukin-6 has also been shown to affect the growth or proliferation of a diversity of hematopoietic cell types in vivo. Ishibashi et al. administered interleukin-6 parenterally to mice and measured platelet counts at various intervals. The results showed that size but not numbers of megakaryocytes was increased and that platelet counts were elevated. No change in myelopoiesis or erythropoiesis was observed. (Ishibashi,T., et al., Blood 74:1241-1244 (1989)). In contrast, other in vivo studies indicated that the administration of interleukin-6 to rats induced myeloid and erythroid hyperplasia (Ulich,T., et al. Blood 73:108-110 (1989); Ulich, T., Am J Path 135:663-670 (1989)).

Neither the in vitro nor the in vivo investigations addressed the mechanism(s) by which interleukin-6 exerts its effects on hematopoiesis. It was not known, until the discovery of the present invention, how interleukin-6 was able to affect erythropoiesis in vivo or in vitro.

SUMMARY OF THE INVENTION

The present invention is based on the inventors' unexpected discovery that interleukin-6 can produce a dramatic increase in the production of endogenous erythropoietin in living cells.

Accordingly, the present invention includes methods for increasing the levels of endogenous erythropoietin in cells in vitro and in vivo by the administration of interleukin-6. The invention is especially directed to methods of enhancing endogenous erythropoietin levels in vivo.

The present invention, thus includes a method of treatment of diseases in which there is a deficiency of erythropoietin, such as the anemias of chronic inflammation, renal failure, AIDs, and malignancy, which method comprises the administration of interleukin-6.

The exemplified methods encompass the enhancement of endogenous erythropoietin levels by living cells in vitro by the administration of interleukin-6 to the cells. In a specific disclosed embodiment, incubation of hypoxic Hep3B human hepatoma cells with interleukin-6 results in a nearly 100% increase in endogenous levels of erythropoietin.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Interleukin Dose-Response Curve in Human Hepatoma Cells. The human hepatoma cell line, Hep3B, which is able to produce biologically active erythropoietin in response tp hypoxia, was used to study the effects of interleukin-6 on erythropoietin production.

Figure 2. Effect of interl ukin-6 on Hypoxia-induced erythropoietin mRNA Levels in Human Hepatoma Cells. Total RNA was extracted from Hep3B cells incubated with or without interleukin-6 in the presence or hypoxia.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relates to the use of interleukin-6 to stimulate the production of endogenous erythropoietin in cells in vitro and in vivo. The invention further includes methods of treatment wherein interleukin-6 is administered to humans and animals having disorders which are manifested by erythropoietin deficiency. The invention also includes methods of enhancing erythropoietin levels in vitro.

In a preferred embodiment, interleukin-6 is administered to humans or veterinary animals in vivo. A preferred in vivo application encompasses the administration of interleukin-6 to humans having disorders that include a deficiency of erythropoietin. Such disorders generally include, but are not limited to, the anemias that accompany renal failure, AIDs, malignancy, and chronic inflammation. Other disorders include anemias not associated with the above conditions such as thalassemia, sickle cell anemia, the anemia of prematurity, anemia that accompanies cis-platinum chemotherapy, and anemia following intensive radiotherapy and/or chemotherapy plus bone marrow transplantation.

In an alternative preferred embodiment of the invention, interleukin-6 is administered to a human

hepatoma cell line maintained under hypoxic conditions.

Other preferred embodiments of the invention include the administration of interleukin-6 derivatives or modifications. Such derivatives or modifications retain the desirable erythropoietin-stimulating activity but may not include amino acid deletions, insertions, substitutions, or modifications.

By the terms "modifications or derivatives," for the purpose of the present invention, is intended any protein or peptide fragment that is functionally-similar to interleukin-6 in that it possesses the activity that enhances the production of erythropoietin and is derivable from the naturally-occurring interleukin-6 molecule. An interleukin-6 peptide derivative or modification is derivable from the naturally-occurring amino acid sequence of interleukin-6 if it can be obtained by fragmenting the naturally-occurring chosen sequence of interleukin-6, or if it can be synthesized based upon a knowledge of the sequence of the naturally-occurring amino acid sequence or of the genetic material (DNA or RNA) which encodes the substance.

By the term "retain", for the purpose of the present invention, is intended, retention, in an interleukin-6 modification or derivative, of erythropoietin-stimulating activity sufficient for the beneficial treatment of a given disorder in which an erythropoietin deficiency is manifested.

By the term "treating" is intended the administration to subjects of the compositions of the invention for purposes which include prophylaxis, amelioration, or cure of disease.

By the term "administer" is intended any method for introducing the compositions of the present invention into a subject. Typical methods include, but are not limited to, oral, intranasal, parenteral (intravenous, intramuscular, or subcutaneous), or rectal. When administration is for the purpose of treatment, administration may be for either a "prophylactic" or "therapeutic" purpose. When provided prophylactically, the substance is provided in advance of any symptom. The prophylactic administration of the substance serves to prevent or attenuate any subsequent symptom. When provided therapeutically, the substance is provided at (or shortly after) the onset of a symptom. The therapeutic administration of the substance serves to attenuate any actual symptom.

By the term "animal" is intended any living creature that contains cells in which erythropoietin production is stimulated in response to interleukin-6 administration. Foremost among such animals are humans; however, the invention is not intended to be so-limiting, it being within the contemplation of the present invention to apply the compositions of the invention to any and all animals which may experience the benefits of the application.

By "enhance" is intended, for the purposes of this invention, an increase in erythropoietin levels to levels greater than those levels that are present before treatment or that result from hypoxia alone.

By the term "disorder" is intended any deviation from or interruption of the normal structure or function of any part, organ, or system (or combination thereof) of the body that is manifested by a characteristic set of symptoms and signs.

By the term "hypoxic" is meant a state characterized by a reduction of oxygen supply below physiological levels. In vivo this state occurs despite the presence of an adequate blood perfusion to the tissues.

EXAMPLES

Example 1

The human hepatoma cell line, Hep3B, which is able to produce biologically active erythropoietin (erythropoietin) in response to hypoxia (Goldberg et al., Proc. Natl. Acad. Sci. USA 84:7972 (1987)) was used to study the effects of Il-6 on erythropoietin production. Hep3B cells were grown to confluency in 100 mm tissue culture dishes (Corning) containing 10 ml of alopha minimal essential medium (Gibco) supplemented with penicillin (100 U/ml), Streptomycin (100 ug/ml), and 10% defined supplemented calf serum (Hyclone). Cells were then incubated under hypoxic conditions (1% oxygen) for 24 hours in triplicate in 5 ml of medium with varying concentrations of Il-6. At the conclusion of the experiment, the culture medium was collected and assayed in duplicate by radioimmunoassay for erythropoietin as described previously (Goldberg et al., Proc. Natl. Acad. Sci. USA 84:7972 (1987)). Values shown represent the mean percent increase in erythropoietin production above that produced by hypoxia alone ± standard deviations. Il-6 was found to cause a dose-dependent increase in the production of erythropoietin in Hep3B cells by as much as 81% at a concentration of 2000 U/ml.

Example 2

Effect of Il-6 on hypoxia-induced erythropoietin mRNA levels. Hep3B cells were grown to confluency in 150 mm culture dishes (Falcon) containing 30 ml of alpha minimal essential medium. Cells were then incubated in 20 ml medium either hypoxically (1% oxygen) or nonhypoxically (21% oxygen) for 24 hours in the presence or absence of Il-6 (1000 U/ml), Il-alpha (20 U/ml) or TNF (1000 U/ml). Total cellular RNA was isolated and an RNA blot analysis was performed as described previously (Goldberg et al., Proc. Natl. Acad. Sci. USA 84:7972 (1987)). Total cellular RNA (20 ug) was loaded in each lane, and the results obtained when the RNA-containing filter was hybridized with 32P-labeled erythropoietin cDNA are shown. As a control, the hybridization to radiolabeled mouse beta-actin is presented at the bottom. In addition, the amount of erythropoietin produced by the cells was determined in duplicate by radioimmunoassay of the culture medium. Using densitometry to scan the blot, it was found that the steady state erythropoietin mRNA levels in the presence of Il-6 were increased 39% above the levels found with hypoxia alone. This indicates that the effect of Il-6 in increasing erythropoietin may occur at the mRNA level; however, the fact that Il-6 caused a 58% increase in total erythropoietin produced by these cells suggests that Il-6 may also have effects at other levels of gene regulation.

Having now generally described this invention, it will become readily apparent to those skilled in the art that many changes and modifications can be made thereto without affecting the spirit or scope thereof.

WHAT IS NEW AND CLAIMED AND INTENDED TO BE COVERED BY LETTERS PATENT OF THE UNITED STATES IS:

1. A method of enhancing erythropoietin production wherein said enhancing is effected by the administration of interleukin-6.

2. The method of claim 1 wherein said interleukin-6 is administered in vitro.

3. The method of claim 2 wherein said administration is under hypoxic conditions.

4. The method of claim 1 wherein said administration is in vivo.

5. A method of treating a disorder in an animal, the pathology of which disorder results from a deficiency of erythropoietin, wherein said treating is by the administration of interleukin-6.

6. The method of claim 5 wherein said disorder is selected from the group consisting of chronic inflammation, renal failure, and cancer.

7. The method of claim 5 wherein said administration is selected from the group consisting of oral, parenteral, nasal, and rectal administration.

8. The method of claim 7 wherein said administration is parenteral.

9. The method of either of claims 1 or 5 wherein said interleukin-6 is in a modified or

derivatized form and wherein said form retains the activity that enhances erythropoietin production.

10. A method of enhancing the production, in cells, of erythropoietin, which method comprises:

administering interleukin-6 to said cells.

FIG. 1

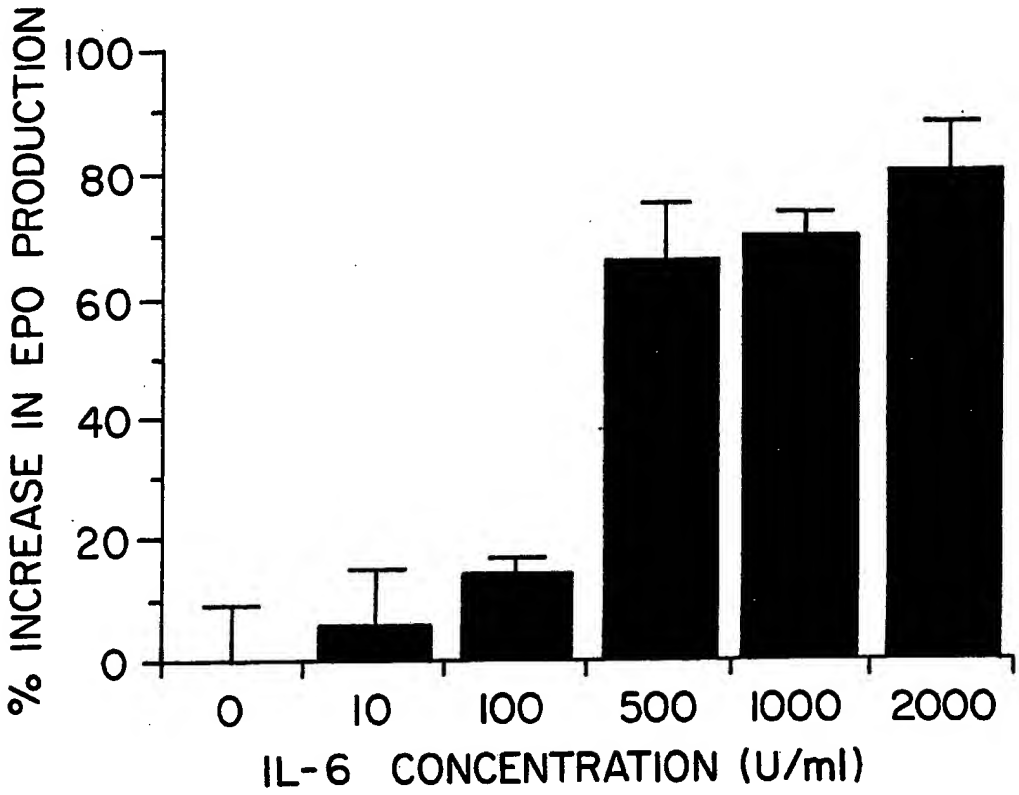
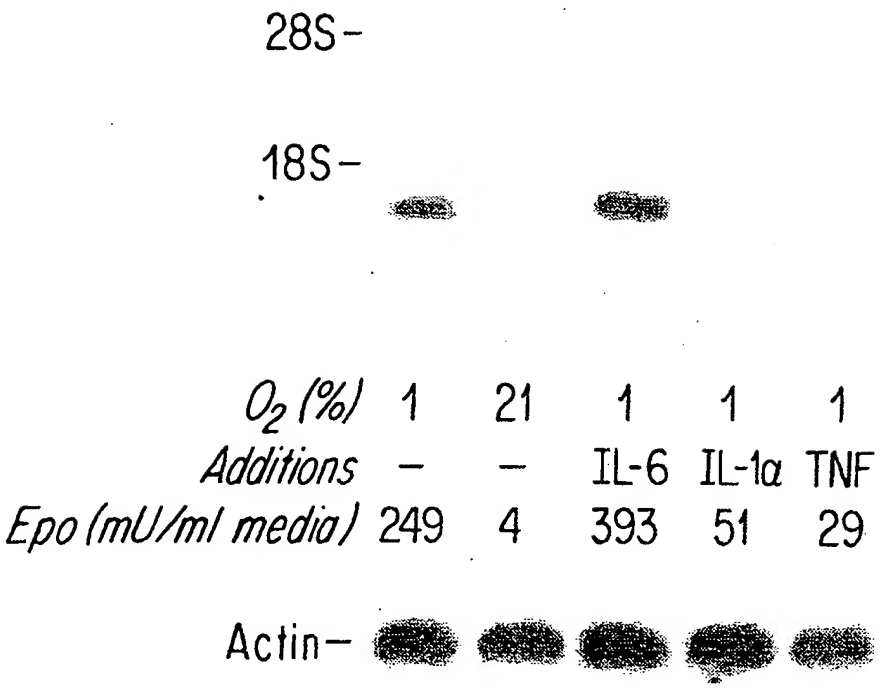

% INCREASE IN EPO PRODUCTION
100
80
60
40
20
0
0
10
100
500
1000
2000
IL-6 CONCENTRATION (U/ml)

FIG. 2


28S-
18S-
O_2 (%) 1 21 1 1 1
Additions – – IL-6 IL-1α TNF
Epo (mU/ml media) 249 4 393 51 29
Actin-

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/08212

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 45/05, 37/10; C07K 13/00
U.S.Cl.: 424/85.2; 530/351; 514/8

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.Cl.	424/85.2; 530/351; 514/8

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

APS, Dialog, search terms: interleukin-6, IL-6, erythropoietin, EPO, anemia, renal, kidney, hypoxia, hypoxic, Hep3B, HepG2

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	The Journal of Immunology, Volume 143, No. 4, issued 15 August 1989, Brakenhoff et al., "Analysis of human IL-6 mutants expressed in Escherichia coli. Biological activities are not affected by deletion of amino acids 1-28", pages 1175-1182, see "Materials and Methods", Table 1.	1-3,9-10 4-8
X Y	The Journal of Biological Chemistry, Volume 263, No. 33, issued 25 November 1988, Baumann et al., "Phorbol ester modulates interleukin 6- and interleukin 1- regulated expression of acute phase plasma proteins in hepatoma cells", pages 17390-17396, see "Materials and Methods", Table 1, Fig. 2.	1-3, 10 4-9
X Y	Blood, Volume 73, No. 1, issued January 1989, Ulich et al., "In vivo hematologic effects of recombinant interleukin-6 on hematopoiesis and circulating numbers of RBCs and WBCs", pages 108-110, see "Materials and Methods".	1, 4-8, 10 2-3, 9

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

22 January 1992

Date of Mailing of this International Search Report

14 FEB 1992

International Searching Authority

ISA/US

Signature of Authorized Officer

Richard Ekstrom

Richard Ekstrom gp

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X Y	Japanese Journal of Cancer Research, Volume 81, No. 10, issued October 1990, Kitahara et al, "The in vivo anti-tumor effect of human recombinant interleukin-6", pages 1032-1038, see abstract, "Materials and Methods".	1, 4-8, 10 2, 3, 9
X Y	The Journal of Experimental Medicine, Volume 171, No. 3, issued 01 March 1990, Mule et al., "Antitumor activity of recombinant interleukin-6 in mice", pages 629-636, see abstract, "Materials and Methods".	1, 4-8, 10 2, 3, 9
A	Proceedings of the National Academy of Sciences U.S.A., Volume 84, issued November 1987, Goldberg et al., "The regulated expression of erythropoietin by two human hepatoma cell lines", pages 7972-7976, see abstract.	1-10